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PRINCIPAL INVESTIGATOR:

Anupama Munshi, Ph.D

CONTRACTING ORGANIZATION:

University of Oklahoma

Oklahoma City, OK 73104

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Anupama Munshi, Ph.D

E-Mail: amunshi@ouhsc.edu

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14. ABSTRACT E-cadherin, is the major adhesion protein associated with epithelial malignancies and loss of E-cadherin expression is diagnostic of EMT in such cells. Loss of E-cadherin plays an important role in breast cancer progression, invasion and metastasis and is used as a prognostic marker for breast cancer. E-cadherin expression is significantly reduced in basal-like and triple negative breast cancers and a higher proportion of E-cadherin aberrations are observed in ER-α negative tumors. Interestingly, E-cadherin, has been shown to interact with ER and studies have demonstrated a direct role for ER-α in controlling E-cadherin expression, with elimination of ER-α from an ER-positive cell line or its reintroduction in an ER-negative context, respectively triggering repression or transcription of E-cadherin. Thus, ER-α may represent the prime factor controlling the expression of this gene in breast cancer cells, an idea previously suggested only by indirect evidence. In addition to a number of relational observations, the absence of ER-α has been mechanistically linked to E-cadherin suppression and EMT and indirect evidence suggests that re-expression of endogenous ER-α is linked to reversion of the invasive breast cancer phenotype. The connection between ER-α and E-cadherin, therefore, is complex and requires a detailed investigation. Several studies have associated loss of ER with worse tumor grade, aggressive biologic behavior and highly radioresistant breast cancer. However no studies have as yet clearly defined the role of E-cadherin in governing radiosensitivity and therefore, in this grant we propose to investigate this connection in greater detail.

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Introduction: Breast cancer is a heterogeneous disease that affects over one million women worldwide every year and includes a wide range of histologic and molecular subtypes that display diverse clinical behaviors. One such subgroup – the "triple negative" (i.e. estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative), includes breast cancers that have undergone an epithelial-to-mesenchymal transition (EMT), are associated with highly invasive clinical disease, have a higher mitotic index, and a worse clinical outcome. E-cadherin, is the major adhesion protein associated with epithelial malignancies and loss of E-cadherin expression is diagnostic of EMT in such cells. Loss of E-cadherin plays an important role in breast cancer progression, invasion and metastasis and is used as a prognostic marker for breast cancer. It has been found that EMT and E-cadherin expression are influenced by several growth factors that are responsive to signaling, as well as by a variety of polypeptide growth pathways. E-cadherin expression is significantly reduced in basal-like and triple negative breast cancers and a higher proportion of E-cadherin aberrations are observed in estrogen receptor-α (ER-α) negative tumors. Interestingly, E-cadherin, has been shown to interact with ER and studies have demonstrated a direct role for ER-α in controlling E-cadherin expression, with elimination of ER-α from an ER-positive cell line or its reintroduction in an ER-negative context, respectively triggering repression or transcription of E-cadherin. Thus, ER-α may represent the prime factor controlling the expression of this gene in breast cancer cells, an idea previously suggested only by indirect evidence. In addition to a number of relational observations, the absence of ER-α has been mechanistically linked to E-cadherin suppression and EMT and indirect evidence suggests that re-expression of endogenous ER-α is linked to reversion of the invasive breast cancer phenotype. The connection between ER-α and E-cadherin, therefore, is complex and requires a detailed investigation. Several studies have associated loss of ER in breast cancer with worse tumor grade and aggressive biologic behavior. Furthermore, lack of ER-α correlates with highly radioresistant breast cancer. However no studies have as yet clearly defined the role of E-cadherin in governing radiosensitivity and therefore, in this grant we propose to investigate this connection in greater detail.

Hypothesis/Rationale/Purpose: The central hypothesis being tested in this project is that EMT determines the radiosensitivity of breast cancer cells. We propose studies to investigate the impact of ER-α and E-cadherin in radiation resistance of breast tumors as well as their role in mediating EMT.

Objectives: Our grant has the following two specific aims:
Aim #1: Does EMT govern the radiosensitivity of breast cancer cells? To understand the functional relationship of ER-α and E-cadherin with respect to mediating EMT and governing radiation response of breast cancer cells? **Aim #2:** Which cell signaling pathway downstream of E-cadherin and ER-α mediates the radioresponse of breast tumor cells?
The subaims under **Aim #1** are listed below:
i). Compare the basal levels of proteins involved in the EMT process including E-cadherin, Vimentin, Snail, Zeb-1 and Zeb-2, Twist, and Slug in a panel of ER-negative and ER-positive human breast cancer cell lines (MDA-MB-231, MCF-7, Hs578t, MDA-MB-468) by Western Blot Analysis.
ii). Compare the intrinsic radiosensitivity of the panel of breast cancer cell lines listed above, using clonogenic cell survival assay.
iii). Test whether re-expressing and stably transfecting breast cancer cells with a plasmid over-expressing E-cadherin in two breast cancer lines, MDA-MB-231 and MDA-MB-468 cells, sensitizes them to radiation. For this we will use a *CDH1* (E-cadherin gene) expression vector from OriGene Technologies Inc. This vector has the cDNA for *CDH1* inserted into the pCMV6-neo vector and has been specifically designed by the company for the purpose of making stable clones. We will transfect

the above mentioned cell lines with this vector using FuGENE 6 transfection reagent. Stable clones will be selected using neomycin (G418). For the control vector, we will excise out the *CDH1* gene and use the re-ligated backbone vector to prepare cells stably expressing the control vector. The ability of E-cadherin to radiosensitize cells upon restoration into the cell lines will be evaluated using clonogenic cell survival assays.

We will also utilize the tetracycline-inducible promoter system to clone E-cadherin. The basic system will be obtained from Clontech. Briefly, we will transfect MDA-MB-231 cells with the pTet-On-Advanced vector and select G418 resistant cells. We will insert the cDNA for *CDH1* into the pTRE-Tight vector and transfected the Tet-On-Advanced MDA-MB-231 cells with the pTRE-Tight-CDH1 response plasmid and the linear hygromycin marker. Hygromycin-resistant cells will be selected and cloned out. Clones will be treated with various doses of doxycycline and tested for E-cadherin expression by western blot. We will extend this approach to test the other E-cadherin negative cell line, MDA-MB-468, in order to extend this to other non-E-cadherin expressing breast cancer lines, i.e. that they are radiosensitized when E-cadherin expression is restored. In each case, parental cells without vector and parental cells containing only the pTet-On-Advanced vector, will be treated with doxycycline to ensure that doxycycline does not affect radiosensitivity. Set up clonogenic cell survival assays to assess radiosensitivity of the above mentioned cell lines.

iv). Test whether knocking down E-cadherin in E-cadherin expressing breast cancer cell lines makes them radioresistant. We will use siRNA approach to downregulate E-cadherin in breast cancer cells and associate downregulation of E-cadherin to radiosensitivity.

We will use siRNA to *CDH1* from Ambion and transfect MCF-7 cells TransIT-TKO transfection reagent. Immunoblot analysis of the transfected cells will be carried out to check for lowered expression of E-cadherin compared to controls over time periods of 1-4 days. Control siRNA, also obtained from Ambion will be used as the control. These preliminary experiments will establish proof-of-principle, i.e. knocking down E-cadherin expression in E-cadherin expressing cells induces radioresistance. The problem with this approach, however, is that the knockdown is only transient, i.e. 3 days, and 3 days may not be sufficient to maximally alter the pathways that mediate radioresistance. As an alternative approach, we will use shRNA expression vectors from OriGene that target *CDH1*. We will isolate stable clones that show reduced expression of E-cadherin. Non-targeting shRNA will also be obtained from OriGene and used as a negative control. Immunoblot analysis to check for suppressed expression of E-cadherin in clones of MCF-7 will be carried out.

The subaims under **Aim #2** are listed below:

i). Our central hypothesis is that the E-cadherin/ER molecular complex governs the radiosensitivity of breast cancer cells through modulation of DSB repair. We will compare the ability of epithelial versus mesenchymal cells to remove DSBs detected as γ-H2AX foci. We will also compare the effect of restoration of E-cadherin expression using a *CDH1*-expression vector in the MDA-MB-231 cells on the kinetics of γ-H2AX foci. On the contrary, we will study the effects of siRNA or shRNA mediated knockdown of E-cadherin on the kinetics of γ-H2AX foci.

ii). We will also test the influence of β-catenin and Zeb1 expression on the ability of E-cadherin to modulate radiosensitivity. When E-cadherin is expressed, β-catenin is sequestered in the cytoplasm and when expression of E-cadherin is lost, β-catenin can locate in the nucleus where it participates in transcription through factors such as Zeb1. We will knock down β-catenin expression in cells lacking E-cadherin using shRNA and testing the influence of this on radiosensitivity. The role of Zeb1 will also be assessed. We will knock down Zeb1 with shRNA and check its effect on radiosensitivity. Since knocking down E-cadherin expression using shRNA induces restoration of Zeb1 expression; Zeb1 then

represses E-cadherin expression in a feed-forward loop. We propose analyses of mesenchymal-like cell lines, i.e. MDA-MB-231 and MDA-MB-468 by knocking down Zeb1 expression using the siRNA and shRNA vectors.

iii). Compare the expression of specific genes/proteins known to modulate EMT by cDNA microarray and RPPA analyses.

Key Research Accomplishments

In the previous cycle we reported that ER positive cells are more epithelial in nature and grow in vitro as organized polygonal cells that maintain cell-to-cell contact, whereas ER-ve cells show a more mesenchymal morphology. These ER-ve cells have undergone a complete EMT which is accompanied by loss of the epithelial marker, E-cadherin and gain of mesenchymal marker, vimentin. Loss of E-cadherin expression is accompanied by overexpression of repressors such as Twist, Zeb1, Snail and Slug. We present some of our western blot data comparing ER positive and ER negative cells for expression of both mesenchymal and epithelial markers (Fig 1). As can be seen from Fig 1, introduction of E-cadherin (CDH1) into MDA-MB-231 and Hs578t cells led to detection of E-cadherin protein and a marked downregulation of the EMT effectors such as Snail and Zeb1. We, however, did not see any obvious changes in Slug or Twist levels in Hs578t (control vs CDH1) and MDA-MB-231 (control vs. CDH1) cells. Surprisingly, we did not see a marked reduction in E-cadherin levels in MCF-7shER cells(MCF-7 cells stably transfected with shRNA to ER) though we could detect induction of the EMT effector, Snail. These effects presumably lead to the suppression/induction of epithelial and mesenchymal genes depending on the model cell system.

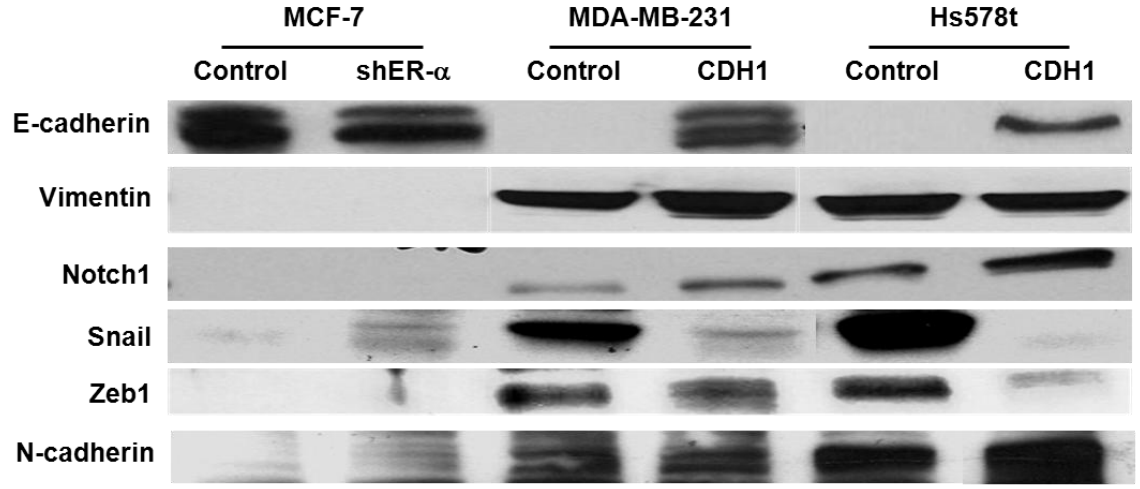

Figure 1: Immunoblot analysis for EMT markers in an ER positive (MCF-7) and ER negative (MDA-MB-231 and Hs578t) breast cancer cell line.

At this point we also obtained a cell line, MCF-M, from Dr. Bruce White at the University of Connecticut Health Center, CT. MCF-7 cells when cultured as mammospheres for 5 weeks underwent a complete EMT. The resulting cell line was labelled as MCF-M and displayed a stable mesenchymal phenotype with a marked increase in EMT-associated transcription factors and decrease in epithelial markers and ER. Since cells which have undergone EMT show an increased motility and proliferation we set up wound healing and cell motility assays to compare the migratory capacity in all our breast cancer cell lines. In scratch wound assays, the parental MCF-7 cells migrated into the wound at a slower rate and failed to close the wound by even 72hrs. In contrast MCF-M cells migrated rapidly and

exhibited closure of the wound as early as 48hr (Fig. 2A). Similar results were obtained upon comparing MCF-7 parental with MCF-shER (Fig 2B). Representative panels from a scratch wound assay were imaged 24 and 48hr post wounding and show slow migration of MCF-7 compared to MCF-shER.

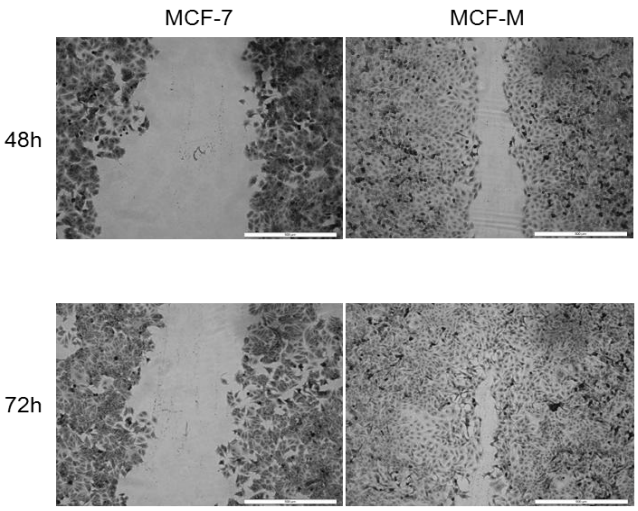

Figure 2A: Gain of migratory ability. Representative panels from a scratch wound assay were imaged 48 and 72hr post wounding and show the slow migration of MCF-7 cells with failure to close the wound, whereas MCF-M cells migrate faster and completely fill the wound.

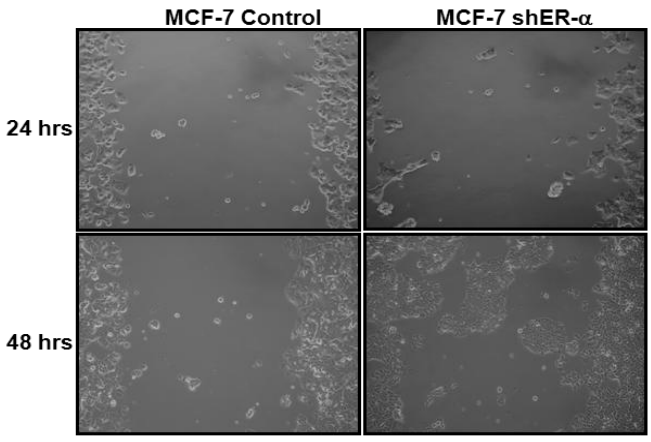

Figure 2B: Gain of migratory ability. Representative panels from a scratch wound assay were imaged 24 and 48hr post wounding and show the slow migration of MCF-7 cells with failure to close the wound, whereas MCF-shER cells migrate faster and start filling the wound by 48hrs.

We further tested the changes in gene expression typically associated with EMT. For this we assayed the expression of EMT proteins in parental MCF-7 and MCF-M cells. We observed a striking decrease in the expression of E-cadherin in MCF-M cells. Additionally, we saw a marked increase in vimentin, Snail and Zeb1 suggesting the acquisition of mesenchymal associated phenotype (Fig. 2C).

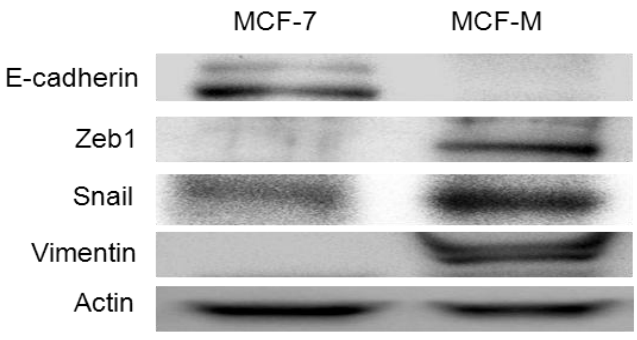

Figure 2C. Comparison of EMT markers in MCF-7 and MCF-M cell lines.

We also carried out an invasion assay on the ER/E-cadherin negative cells (MB231 and Hs578t) cells and compared them to the E-cadherin overexpressing clones. Additionally, MCF-7 cell were compared to the MCF-shER cells. MCF-shER cells were highly invasive compared to the parental MCF-7 cells (Fig. 3). The MDA-MB-231 CDH1 cells did not show any significant difference in invasive capacity compared to the parental 231 whereas the Hs578t cells overexpressing CDH1 were less invasive compared to the parental 578t cells (Fig 3).

To correlate the loss of ER with E-cadherin we used the MCF-shER cells in which knockdown of ER expression results in a loss of E-cadherin as well. Since MCF-7 cells, (ER and E-cadherin positive) are very radiosensitive we decided to test the correlation between loss of ER and E-cadherin and radiation sensitivity. MCF-7 control cells were compared with MCF-7 shER for their radiation response by clonogenic cell survival assays. MCF-7 shER stable transfectants demonstrated an increased resistance to radiation when compared with the vector control cells indicating that knockdown

of ER imparted a radiation resistant phenotype to the cells (Fig 4). Additionally, we also prepared MDA-MB-231 stable cells overexpressing E-cadherin and set up clonogenic cell survival experiments to compare the radiation response of MDA-MB-231-CMV control cells versus MDA-MB-231-CDH1. Overexpression of E-cadherin made the MB-231 cells sensitive to radiation as can be seen in Fig 4. Similar results were obtained using Hs578t cells stably expressing E-cadherin (Fig 4). All clonogenic assays were repeated at least three times to ensure reproducibility and obtain statistically significant data. Our results demonstrate a role for ER and E-cadherin in controlling radiation response.

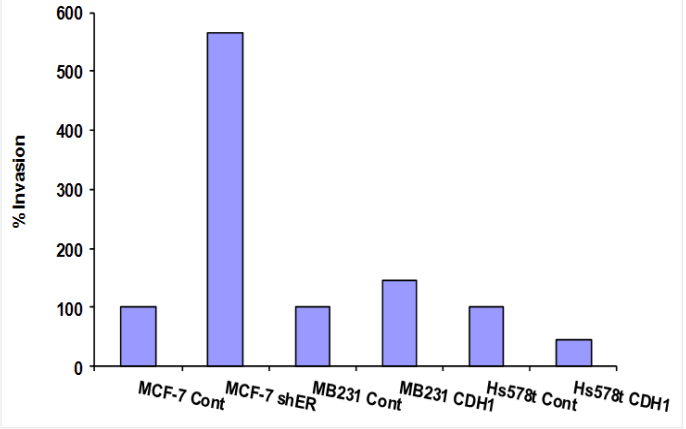

Figure 3: Loss of ER and gain of E-cadherin imparts an invasive phenotype.

Clonogenic assays were also set up to determine the radiosensitivity of the MCF-M cells compared to the parental MCF-7 cells. Though preliminary, our data demonstrates that MCF-M cells that have undergone EMT are more radioresistant when compared to the parental MCF-7 cells which are epithelial in nature (Fig. 5).

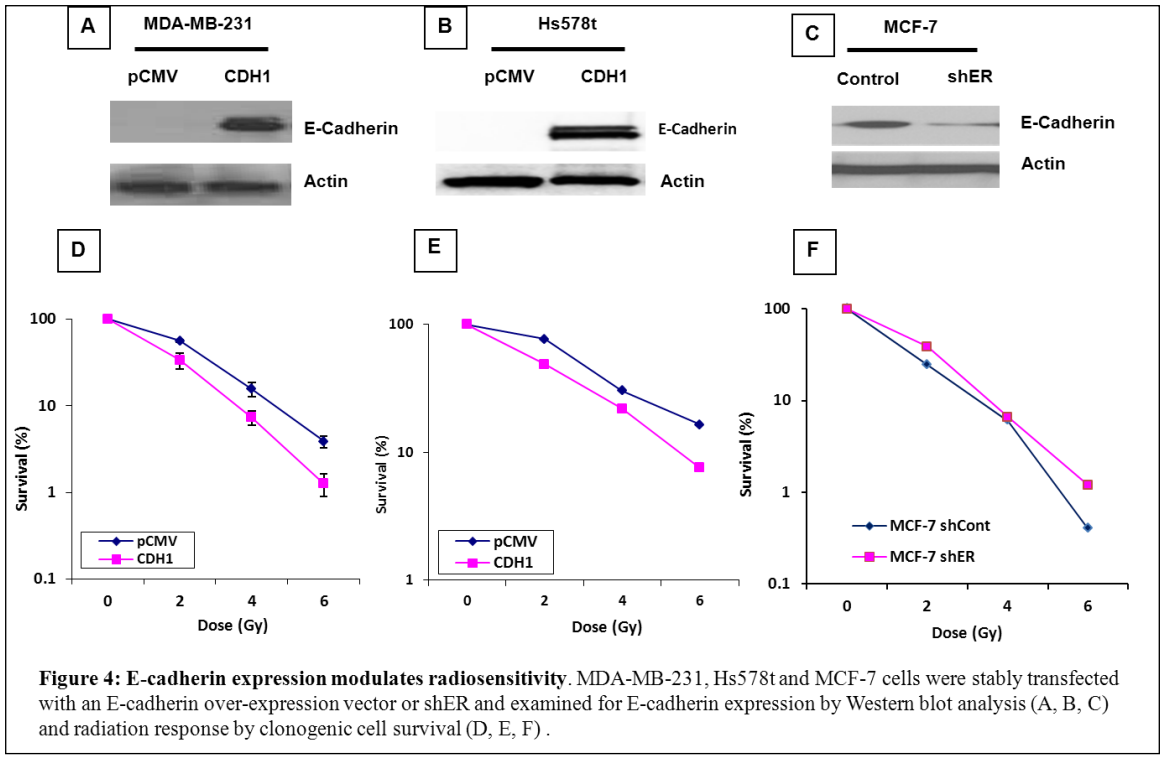

Figure 4: E-cadherin expression modulates radiosensitivity. MDA-MB-231, Hs578t and MCF-7 cells were stably transfected with an E-cadherin over-expression vector or shER and examined for E-cadherin expression by Western blot analysis (A, B, C) and radiation response by clonogenic cell survival (D, E, F) .

The EMT process is also known to confer another welcome trait to cancer cells- expansion of the cancer stem cell fraction. The CSC subpopulation retains tumorigenic and self-renewal potential and is relatively insensitive to anti-tumor therapies. Thus, EMT increases cancer metastasis and the probability of recurrence.

Mammosphere culture and EMT have been shown to enrich for ALDH1+/CD44+/CD24- stem cell phenotype in breast cancer cells. Thus, we compared ALDH1 expression in ER positive (MCF-7) and ER negative (MDA-MB-231 and Hs578t) cell lines. MDA-MB-231 and Hs578t cells had higher ALDH1+ cells when compared with the MCF-7 cells, consistent with a cancer stem cell phenotype (Fig. 6). Our preliminary findings indicate that ER negative cells are enriched for cancer stem cells and this could be a potential cause for the radiation resistance of ER negative cell lines. We conducted additional studies to include MCF-shER and the CDH1 overexpressing MDA-MB-231 for ALDH1+ expression. As can be seen from Fig. 7, knockdown of ER in MCF-7 cells led to an increase in the ALDH1+ population. Similarly, we observed a slight decrease in the ALDH1+ cells in MDA-MB-231 CDH1 compared to the parental MB-231 cells (Fig. 7). These results, though preliminary, indicate a link between ER, E-cadherin, EMT and the cancer stem cell phenotype- all factors that can dictate the response to radiation. To better address these results we are conducting a detailed investigation.

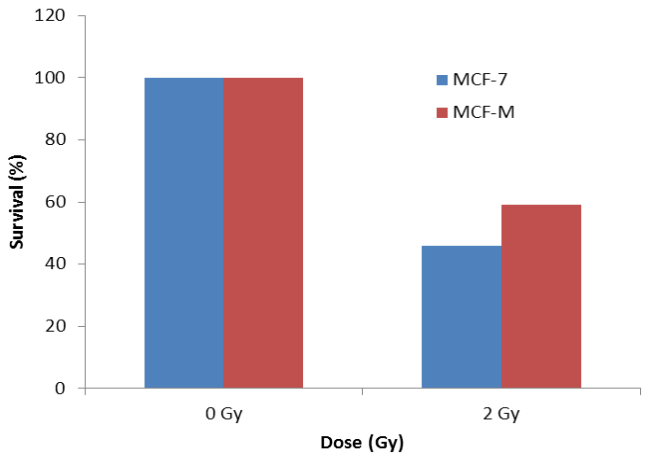

Figure 5: Clonogenic assay comparing the radiation response of MCF-7 with MCF-M cells.

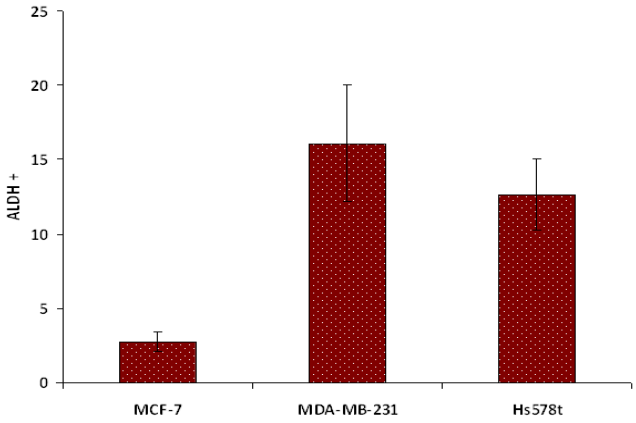

Figure 6: Aldefluor assay on breast cancer lines

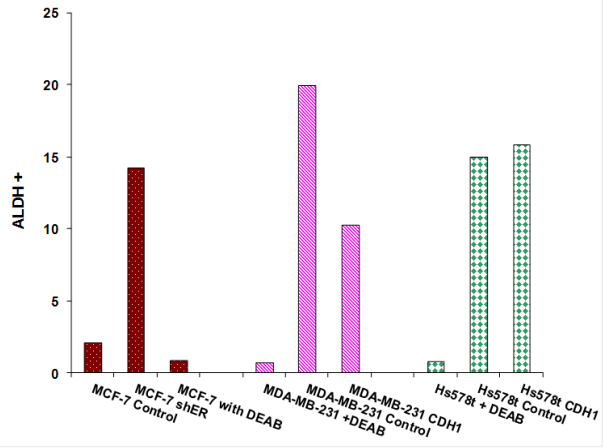

Figure 7: Aldefluor assay comparing ALDH1+ cells in a panel of breast cancer cell lines.

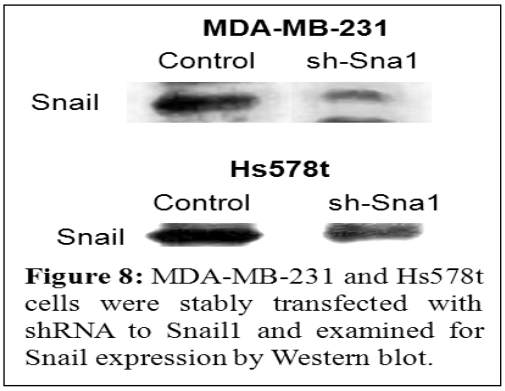

Figure 8: MDA-MB-231 and Hs578t cells were stably transfected with shRNA to Snail1 and examined for Snail expression by Western blot.

As part of Aim #2 we had proposed to compare the ability of epithelial versus mesenchymal cells to remove DSBs detected as γ-H2AX foci. We have begun an initial study to compare the levels of DNA repair proteins as well as kinetics of H2AX foci in our panel of cell lines that have been used during the course of the study.

Additionally, based on the western data shown in Fig. 1, it seems that modulation of E-cadherin alters the levels of Snail and Zeb1 in the CDH1 over-expressing MDA-MB-231 and Hs578t cells. Since knocking down E-cadherin expression using shRNA induces restoration of Zeb1 expression; Zeb1 then represses E-cadherin expression in a feed-forward loop, we will investigate the

effect of downregulation of Zeb1 and Snail in radiation sensitization of our breast cancer cell lines. To that end, we have prepared MDA-MB-231 and Hs578t cells in which we have knocked down Zeb1 as well as Snail1 with shRNA (results shown for Snail 1 knockdown) (Fig. 8). These cells are in the process of being evaluated for their radiation response.

A sub-part of Aim #2 was to compare the expression of specific genes/proteins known to modulate EMT by cDNA microarray and RPPA analyses. We have been able to successfully complete the RPPA analyses on our panel of breast cancer lines and present some of the data below. As can be seen from Fig. 9 the MCF-7 epithelial line showed high expression of E-cadherin as compared to the "mesenchymal-like" MDA-MB-231 and Hs578t cells. Conversely, the mesenchymal cells demonstrated increased expression of the mesenchymal markers – Fibronectin, caveolin, Snail and β-catenin. These results have been confirmed by western blot analysis (data not shown). Additionally, we are in the process of analyzing all the RPPA data that we have collected on MDA-MB-231 CDH1, MDA-MB-231 sh-Snail, MDA-MB-231 shZeb1, Hs578t-CDH1, Hs578t-shZeb1, Hs578t-shSna1, MCF-M and MCF-shER.

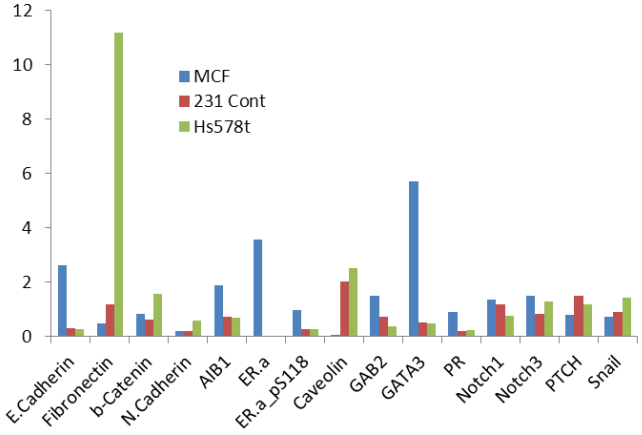

Figure 9: RPPA analyses for EMT markers in an ER positive (MCF-7) and ER negative (MDA-MB-231 and Hs578t) breast cancer cell line.

The data will be presented upon completion of analysis.